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Alpha-Fetoprotein Controls Female Fertility and Prenatal Development of the Gonadotropin-Releasing Hormone Pathway through an Antiestrogenic Action†

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It has been shown previously that female mice homozygous for an alpha-fetoprotein (AFP) null allele are sterile as a result of anovulation, probably due to a defect in the hypothalamic-pituitary axis. Here we show that these female mice exhibit specific anomalies in the expression of numerous genes in the pituitary, including genes involved in the gonadotropin-releasing hormone pathway, which are underexpressed. In the hypothalamus, the gonadotropin-releasing hormone gene, *Gnrh1*, was also found to be down-regulated. However, pituitary gene expression could be normalized and fertility could be rescued by blocking prenatal estrogen synthesis using an aromatase inhibitor. These results show that AFP protects the developing female brain from the adverse effects of prenatal estrogen exposure and clarify a long-running debate on the role of this fetal protein in brain sexual differentiation.

The alpha-fetoprotein (AFP) gene is a member of the albumin gene family and encodes a serum glycoprotein with an oncofetal pattern of expression. AFP is produced in high concentrations during embryonic life by the hepatocytes and the visceral endoderm of the yolk sac and to a lesser extent by the developing gastrointestinal tract and kidney (2, 26, 39). Its synthesis decreases dramatically shortly after birth to reach trace amounts a few weeks later but can be restored during life when liver pathologies or some types of tumors develop (hepatitis, cirrhosis, hepatoma, teratocarcinoma, and some pancreatic and renal tumors) (1, 11, 26, 27, 31, 39).

The exact function of AFP has been the subject of a long-running debate. One important feature of AFP is its capacity to bind estrogens, but not androgens, at its C-terminal extremity with a K_a of 10^9 M^{-1} (33, 35, 43), indicating that it can act as an estrogen carrier in the blood. Although human AFP has not been demonstrated to bind estrogens, human AFP peptides do so and human AFP possesses an antiestrogenic activity (7, 44). Human AFP could thus be involved in antiestrogenic effects, just like rodent AFP. Because perinatal exposure to estrogens in rodent females results in anovulatory sterility associated with altered gonadotropin production (16, 23, 29), it is classically assumed that the function of AFP is to sequester circulating estrogens and, by so doing, to protect the developing female brain from their effects (for a review, see reference 32). Alternatively, because AFP is found inside neurons without being produced locally, it has been suggested that AFP has more than a passive neuroprotective role and specifically delivers estrogens into certain brain cells in order to ensure correct female brain differentiation (18, 41).

Afp gene knockout (AFP KO) mice have been generated by Gabant and coworkers (21). The mice homozygous for the targeted allele are viable and develop normally, but females are sterile due to anovulation. Reciprocal transfer experiments with ovarian tissue have demonstrated that the ovaries are functional but lack an adequate signal from the hypothalamic-pituitary axis to execute ovulation. To determine which pathways of the hypothalamic-pituitary-gonadal axis are altered in adult female AFP KO mice, we compared the gene expression profiles in such females and their normal counterparts by microarray analysis. Furthermore, these knockout animals allowed us to determine whether AFP has only a neuroprotective role (passive estrogen carrier) or not. We reasoned that if AFP was essentially a passive estrogen carrier, the fertility of the AFP KO females should be restored if their embryonic development could take place in the absence of estrogens. Estrogen-free pregnancy can be achieved by treating pregnant females with an aromatase inhibitor (8). We have thus determined whether the fertility of AFP KO females that developed in an estrogen-free environment was rescued, as well as whether their gene expression profiles in the hypothalamic-pituitary-gonadal axis were normalized.

MATERIALS AND METHODS

Microarray study. (i) Animals. AFP KO mice have been described previously (21). The CD1 animals used in the microarray analysis were bearing the $Afp^{tm2Ibmm}$ allele (knockout 2, described in reference 21) and were from the fifth

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backcross generation. Mice were housed under a 12-h light/dark cycle (lights off at 6:30 p.m.). Food and water (Scientific Animal Food and Engineering, Augy, France) were available ad libitum. Animals were killed by cervical dislocation between the ages of 4 and 5 months. Females were in the metoestrus II/dioestrus phases of the estrus cycle as established by vaginal smears, according to our previous observation that AFP KO females remain in these phases (21). The pituitaries were dissected in one piece while a 4-mm^3 side piece was cut around the pituitary stalk in the brain, permitting dissection of a zone containing the hypothalamus. Tissues were immediately processed after dissection by disruption in a Dounce homogenizer in the presence of TRIzol reagent (Life Technologies, Inc., Carlsbad, CA). Total RNA was further extracted according to the TRIzol manufacturer's instructions and resuspended in RNA storage solution (Ambion Inc., TX).

(ii) RNA preparation and Affymetrix GeneChip hybridization. The quality of the RNAs was checked with an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). All the RNAs used in the experiment met the quality criteria defined by Agilent. Genes expressed in each sample were analyzed on a high-density oligonucleotide microarray (MOE-430A; Affymetrix, Santa Clara, CA) containing 22,690 transcripts. Target preparation and microarray processing procedures were performed as described in the Affymetrix *GeneChip Expression Analysis Manual*. Briefly, 3 μg of total RNA was used to synthesize double-stranded cDNA with SuperScript II reverse transcriptase (Life Technologies, Inc., Rockville, MD) and a T7-$(dT)_{24}$ primer (Proligo, Paris, France). Then, biotinylated cRNA was synthesized from the double-stranded cDNA with the RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY) and was purified and fragmented. The fragmented cRNA was hybridized into the oligonucleotide microarray, which was washed and stained with streptavidin-phycoerythrin. Scanning was performed with an Agilent microarray scanner.

(iii) Data analysis. All the "dat" files (corresponding to the scan image) and the "rpt" files (corresponding to each GeneChip) were first checked for the control of hybridization defined by the Affymetrix GeneChip manual (uniformity of the signal, good position of the grid, scale factor, background, percentage of presence, and presence of housekeeping genes and spike control). All experiments were also analyzed with the "deg" algorithm associated with Bioconductor 1.3 software (http://www.bioconductor.org/). This algorithm analyzes the quality of the mRNA by comparing the signal intensities in the 5′ and 3′ ends of the mRNA, which should be similar if no RNA degradation has occurred. All quality controls were positive and are available in Table S1 in the supplemental material.

We also conducted an analysis using robust microarray analysis (RMA), which first calculates the probe-specific correction of the perfect-match probes using a model based on observed intensity being the sum of signal and noise and then normalizes the perfect-match probes by quantile normalization. The expression measure was calculated using a median polish (24, 25). The "Cel" files of each experiment were collected with Bioconductor 1.3 software and treated according to the RMA package (we used quantile normalization). All data were then collected by BRB 3.1 software, developed by Richard Simon and Amy Peng Lam (http://linus.nci.nih.gov/BRB-ArrayTools.html). A list of genes was generated using the Class Comparison Tool associated with BRB 3.1. Because of the small sample size, a randomized-variance *t* test was used with a threshold level of significance (*P*) of <0.05. In addition, an FDR ("false discovery rate") correction procedure was applied using a *P* value of 0.05 (5, 6).

(iv) Real-time PCR analysis. Results obtained by the microarray technology were confirmed with reverse transcription and quantitative PCR amplification (quantitative RT-PCR) technology. Results from the pituitary were confirmed with 7900HT microfluidic card (Applied Biosystems, Foster City, CA) technology. For each group of animals (wild-type [WT] and knockout females), we used two mRNA samples that were previously analyzed with microarray technology and an additional one not analyzed before. RNA (1 μg) from each animal was retrotranscribed with the high capacity cDNA archive kit (Applied Biosystems) in a total volume of 10 μl. Then, 1 μl of the RT product (100 ng cDNA) was loaded into each well of the 7900HT microfluidic card (Applied Biosystems) for quantitative PCR amplification with universal PCR master mix (Applied Biosystems). Data were analyzed by the relative quantification software available on the machine. The housekeeping gene *Hprt* (hypoxanthine guanine phosphoribosyl transferase) was used as a reference gene for normalization. In the case of the *Gnrh1* gene (gonadotropin-releasing hormone), the differential expression between normal and knockout females ($n = 7$ [each genotype]) was tested on the 7300 real-time PCR system (Applied Biosystems) with the absolute quantification software. Total RNA (1 μg) was retrotranscribed with the high capacity cDNA archive kit (Applied Biosystems), and 100 ng of cDNA was brought in the PCR mixture. The housekeeping gene *Hprt* was used as reference gene for normalization. PCR was performed with qPCR MasterMix for SYBR Green I (Eurogentec, Seraing, Belgium).

Aromatase inhibitor treatment. Females heterozygous for the $Afp^{tm2Ibmm}$ allele (CD1 strain, eighth backcross level) were mated with $Afp^{tm2Ibmm}$ homozygous males and housed under the conditions described above. They then received a daily subcutaneous injection in the neck of 4 mg of the aromatase inhibitor ATD (1,4,6-androstatrien-3,17-dione; Steraloids, Newport, Rhode Island) dissolved in propylene glycol, from day 13.5 of gestation (day 0.5 was defined as the day of vaginal plug detection) until the end of gestation. Controls were injected with propylene glycol only. Pups were born naturally or retrieved by cesarean operation on embryonic day 20.5. Blood from the mothers was taken from the heart, and estradiol levels were measured by classical radioimmunoassay. Fertility of female pups was tested at the age of 2 to 3 months. In parallel, sisters of the female pups tested were dissected at the age of 3 months, and total RNA was extracted from the pituitary and the hypothalamus as described above. cDNA was obtained using the TaqMan reverse transcription reagent kit (Applied Biosystems), and real-time PCR was performed on the 7300 real-time PCR system (Applied Biosystems) with the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen) and TaqMan Gene Expression Assays (Applied Biosystems) for pituitary genes except for *Egr1*. The *Gnrh1* and *Egr1* transcripts were quantified with the same machine and software but with the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, California) for PCR. Statistical testing for pairwise comparison between groups was made with the *t* test ($\alpha = 0.05$) or with the Mann-Whitney rank sum test.

RESULTS

Gene expression profile in the AFP KO female hypothalamic-pituitary axis. Total RNA from the pituitary of phenotypically normal WT mice and AFP KO mice ($n = 3$ [each group]) was analyzed by Affymetrix microarray technology on mouse expression set 430A. We first compared the WT females to the AFP KO females. In order to reduce the background generated by genes whose expression levels were very close to the median expression level, we first filtered data by choosing the genes showing a variance of the log ratio significantly different from the median of all the variances ($P = 0.01$). Using this step, a total of 2,676 probe sets were selected. We then used a nonpaired randomized-variance *t* test with a *P* value of 0.05 (because of the small size of the samples) (4). This test generated a list of 1,392 differently expressed probe sets associated with the AFP KO phenotype. As described in Materials and Methods, an FDR procedure (5, 6) was applied to correct the *P* value for multiple comparisons, and 929 probe sets showing differential expression were validated with a *P* value of 0.05. Complete results are summarized in Table S2 in the supplemental material. These genes define the AFP KO female signature. The extreme values were 0.203 for the lower range (*Fos* [FBJ osteosarcoma oncogene]) and 4.464 for the upper range (epidermal arachidonate lipoxygenase). In this list, 47 probe sets corresponding to 39 different genes (because of the redundancy of probe sets) showed a minimum twofold change. They are listed in Table 1. We also compared the WT males to the AFP KO males. No anomalies were detected in the AFP KO males; thus, the male pituitaries were not further analyzed.

The microarray results obtained for female animals were then validated by quantitative RT-PCR experiments. Among the 929 genes showing differential expression (and listed in Table S2 in the supplemental material), 34 genes were selected according to two criteria—their biological relevance in fertility pathways (GnRH receptor pathway, lactation) or in neuronal activity and their differences in expression level—to cover a wide range of up- and down-regulation in the AFP KO female pituitaries. The results are shown in Table 1 (12 genes showing a minimum twofold change in the microarray analysis and 22 other genes among the 34 selected genes). A total of 33 of the

TABLE 1. Selected genes differentially expressed in female AFP KO pituitaries

Affymetrix probe set	Unigene no.	Gene symbol	Fold geometric mean change as established by[a]:	
			Microarray	RT-PCR
Genes differentially expressed by a factor of ≥2 (microarray analysis)				
1423100_at	Mm.246513	*Fos*	0.203	0.462
1417025_at	Mm.22564	*H2-Eb1*	0.277	
1450407_a_at	Mm.269088	*Anp32a*	0.278	
1418188_a_at	Mm.358667	*Ramp2*	0.281	
1421665_a_at	Mm.26791	*Gnrhr*	0.285	0.588
1416236_a_at	Mm.33240	*Eva1*	0.310	0.535
1424609_a_at	Mm.182434	*Fstl1*	0.317	
1448107_x_at	Mm.142722	*Klk6*	0.327	
1424607_a_at	Mm.181959	*Egr1*	0.348	0.628
1456341_a_at	Mm.291595	*Bteb1*	0.351	
1427104_at	Mm.87487	*Zfp612*	0.378	
1438676_at	Mm.275893	*Mpa21*	0.391	
1422809_at	Mm.309296	*Rims2*	0.400	0.552
1454607_s_at	Mm.289936	*Psat1*	0.405	
1455988_a_at	Mm.153159	*Cct6a*	0.407	
1448830_at	Mm.239041	*Dusp1*	0.409	
1417513_at	Mm.35796	*Evi5*	0.413	
1418908_at	Mm.5121	*Pam*	0.423	
1423804_a_at	Mm.29847	*Idi1*	0.426	
1437082_at	Mm.46044	*Akap9*	0.442	
1423619_at	Mm.3903	*Rasd1*	0.449	
1456175_a_at	Mm.261735	*Copb2*	0.454	
1416131_s_at	Mm.260647	*C920006C10Rik*	0.462	
1434113_a_at	Mm.252772	*Xpmc2h*	0.472	
1429296_at	Mm.74596	*Rab10*	0.475	
1426956_a_at	Mm.215389	*Trp53bp1*	0.476	
1452377_at	Mm.2389	*Mll*	0.478	
1434578_x_at	Mm.297440	*Ran*	0.485	
1433446_at	Mm.61526	*Hmgcs1*	0.490	0.593
1434380_at	Mm.254851	*Dnr12*	0.498	
1455141_at	Mm.218873	*Tnrc6a*	0.500	
1448312_at	Mm.294493	*Pcsk2*	2.012	1.729
1420575_at	Mm.2064	*Mt3*	2.141	1.521
1428014_at	Mm.158272	*Hist1h4h*	2.252	
1449992_at	Mm.207082	*Isp2*-pending	2.611	35.750
1430111_a_at	Mm.4606	*Bcat1*	2.924	
1418197_at	Mm.4177	*Ucp1*	3.049	132.265
1420338_at	Mm.4584	*Alox15*	3.067	75.309
1426039_a_at	Mm.274093	*Alox12e*	4.464	31.213
Additional genes tested by quantitative RT-PCR				
1450033_a_at	Mm.277406	*Stat1*	0.554	0.398
1422629_s_at	Mm.46014	*Shrm*	0.563	0.546
1451718_at	Mm.1268	*Plp*	0.567	0.342
1427683_at	Mm.290421	*Egr2*	0.570	0.620
1424470_a_at	Mm.24028	*9330170P05Rik*	0.592	0.607
1450646_at	Mm.140158	*Cyp51*	0.606	0.655
1421191_s_at	Mm.142822	*Gopc*	0.634	0.636
1452036_a_at	Mm.159684	*Tmpo*	0.643	0.646
1417385_at	Mm.29824	*Psa*	0.652	0.905
1426819_at	Mm.248335	*Fosb*	0.658	0.464
1460248_at	Mm.10233	*Cpxm2*	0.663	0.589
1417444_at	Mm.153415	*E2f5*	0.714	0.644
1460329_at	Mm.26364	*B4galt6*	0.756	0.642
1448754_at	Mm.279741	*Rbp1*	0.769	0.462
1451695_a_at	Mm.332810	*Gpx4*	1.391	1.483
1419469_at	Mm.139192	*Gnb4*	1.499	1.519
1424171_a_at	Mm.43784	*Hagh*	1.543	1.499
1417111_at	Mm.117294	*Man1a*	1.546	2.540
1417502_at	Mm.18590	*Tm4sf2*	1.590	2.192
1422557_s_at	Mm.192991	*Mt1*	1.645	1.554
1422586_at	Mm.140765	*Ecel1*	1.815	2.948
1451342_at	Mm.334160	*Spon1*	1.946	5.139

[a] Values are ratios of AFP KO to WT mice.

34 selected genes also showed differential expression between WT and AFP KO females (the only exception was the *Psa* gene), thereby largely confirming the results of the microarray analysis. It is notable that among the genes defining the AFP KO female signature (see Table S2 in the supplemental material), several were previously reported to be important in female fertility, on the basis of the phenotype observed in the corresponding knockout mouse models, namely, *Egr1* (28), *Cish2* (9), *Ptprf* (36), *Psa* (34), and *Tkt* (47).

As reported previously, AFP KO females show an anovulatory phenotype (21). The GnRH receptor pathway is particularly important for ovulation, and this pathway is disturbed in AFP KO females. Indeed, the GnRH receptor mRNA level is reduced by factors of 3.5 (microarrays) to 1.7 (quantitative RT-PCR) in the AFP KO females compared to the WT females (Table 1; see also Table S2 in the supplemental material). Several downstream genes are also down-regulated, as described below.

The GnRH receptor is a seven-transmembrane-domain G protein-coupled receptor. The number of these receptor molecules varies over the estrus cycle and is thought to correlate with the gonadotropin secretory capacity of the pituitary gonadotroph cells (13). It is also correlated with the level and frequency of release (pulsatility) of its ligand, the decapeptide GnRH produced by the hypothalamus (12, 15, 30). Binding of GnRH to its receptor induces a rise in intracellular Ca^{2+} concentration and activates the diacylglycerol-protein kinase C pathway (for a review, see reference 10). This pathway directly influences the Egr1 transcription factor that binds to the luteinizing hormone beta subunit gene (*Lhb*) promoter, thereby activating its transcription. When the GnRH receptor is activated, the Egr1 mRNA level increases and the activation effect of Egr1 on the *Lhb* promoter is further enhanced by protein kinase C-elicited phosphorylation (19, 42). In accordance with a down-regulation of the GnRH receptor gene in the AFP KO female pituitary, the Egr1 mRNA is down-regulated in these mice (Table 1 and Fig. 1). Furthermore, *Fos*, the mRNA level of which correlates with that of *Egr1* (38), is also reduced (Table 1 and Fig. 1). Interestingly, we detected that the expression level of *Fosb* is also down-regulated in the AFP KO female pituitary, suggesting that both *Fos* and *Fosb* could be regulated by *Egr1*. In addition, we observed differences in the expression levels of three other genes previously described as being part of the GnRH receptor-coupled gene network (45), namely, transforming growth factor β1-induced transcript 4 (*Tgfb1i4*), protein tyrosine phosphatase 4a1 (*Ptp4a1*), and early growth response 2 (*Egr2*) genes. The first two genes are down-regulated in AFP KO females by a factor of 1.3 (Fig. 1; see also Table S2 in the supplemental material), and *Egr2* is down-regulated by a factor of 1.7.

On the other hand, other hormonal secretions of the pituitary represented on mouse expression set 430A showed no differences in expression levels between WT females and AFP KO females, namely, *Gh* (growth hormone), *Prl* (prolactin), *Fsh* (follicle-stimulating hormone), and *Pomc* (pro-opiomelanocortin). Thus, differential expression of pituitary hormonal transcripts between WT and AFP KO females seems to be limited to the GnRH receptor pathway.

Since the GnRH receptor level is down-regulated in the AFP KO female pituitary, and since this level is known to be linked to the level of its ligand in a dose-dependent manner (15), we decided to quantify the gonadotropin-releasing hormone gene (*Gnrh1*) mRNA level in the hypothalamus (by quantitative RT-PCR with SYBR Green dye). *Gnrh1* mRNA was found to be down-regulated by a factor of 2.5 in the AFP KO female hypothalamus (Fig. 1) (no difference was found between WT and AFP KO males).

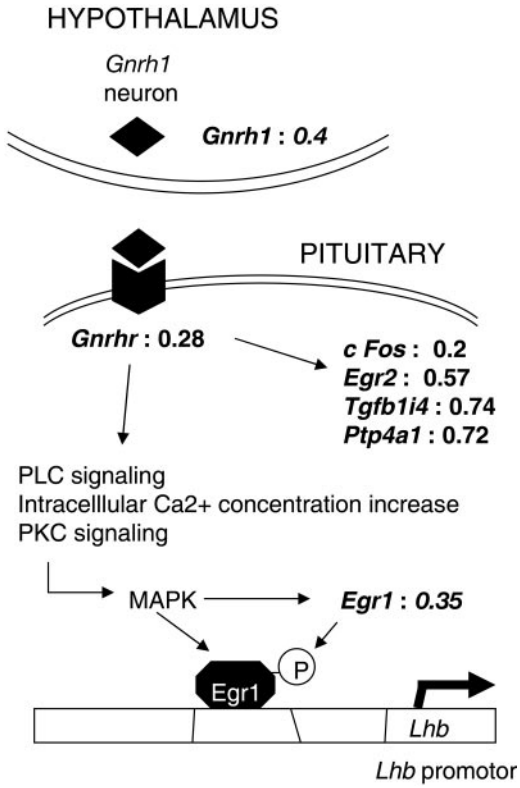

FIG. 1. Schematic view of the GnRH pathway. Genes whose expression is affected in AFP KO females are indicated in bold print. Changes in mRNA levels are shown as ratios of AFP KO to WT levels.

Estrogen-free embryo development rescues fertility in AFP KO females. (i) Fertility. AFP KO females are sterile either because AFP could not play its neuroprotective role against the effects of circulating estrogens or because AFP could not actively bring estrogens to specific brain cells. In order to discriminate between these two hypotheses, we injected the aromatase inhibitor ATD to block estrogen synthesis in heterozygous females during the late gestational period, which is a critical period for brain sexual differentiation. The former hypothesis predicts that AFP KO female fetuses which develop in an estrogen-free environment should be fertile (no estrogen, no need for protective AFP). In contrast, the latter hypothesis predicts that AFP KO females should be sterile (no estrogen carrier AFP). Serum estradiol levels of the ATD-treated gestating females proved the effectiveness of the treatment (estradiol levels were below the 10-pg/ml detection limit of radioimmunoassay).

Heterozygous gestating females (13 animals) were injected with ATD, and a total of 17 AFP KO female pups and 25 heterozygous female pups were retrieved. The fertility of 8 of the AFP KO pups and of 17 of the heterozygous pups was

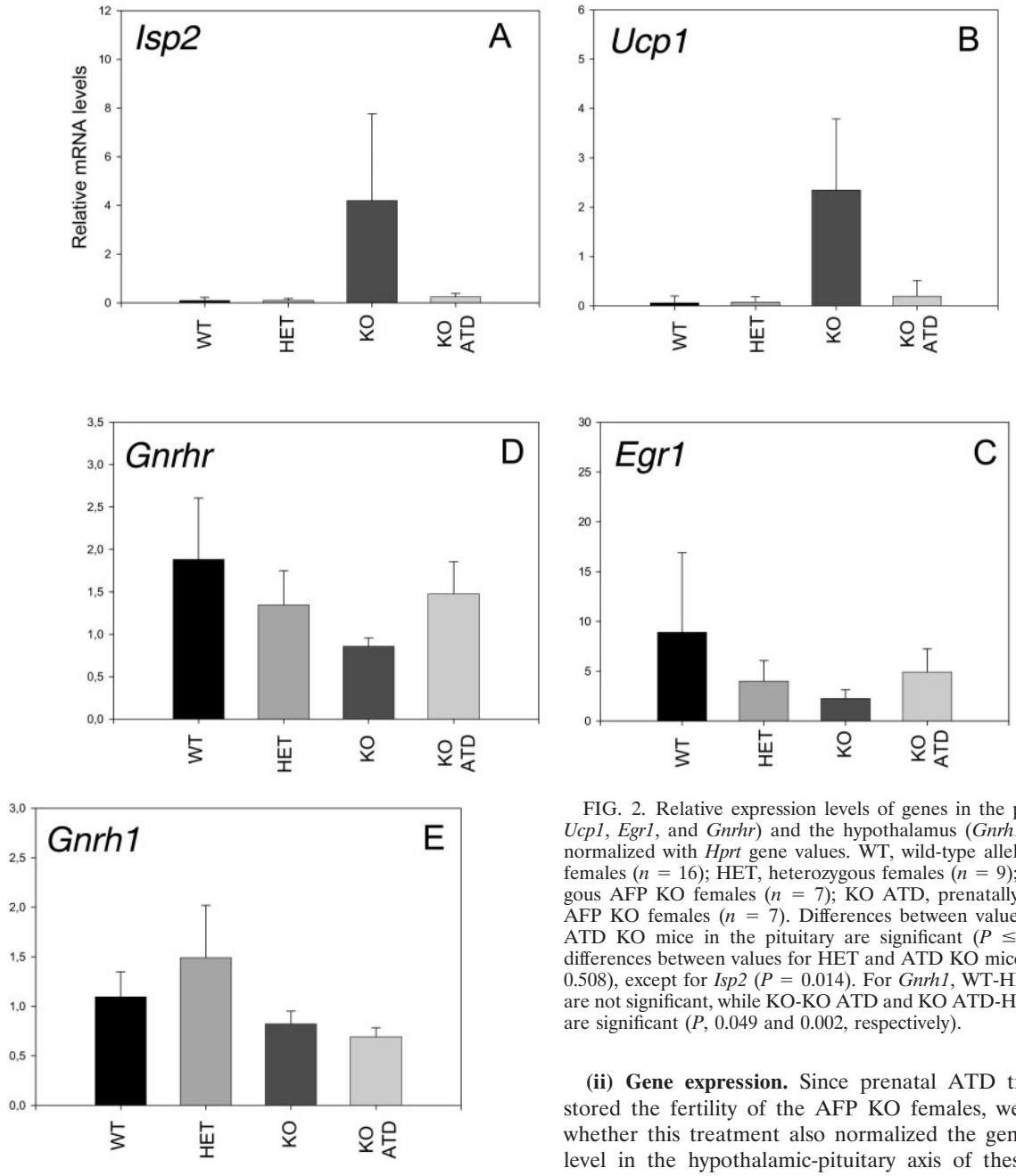

FIG. 2. Relative expression levels of genes in the pituitary (*Isp2*, *Ucp1*, *Egr1*, and *Gnrhr*) and the hypothalamus (*Gnrh1*). Values are normalized with *Hprt* gene values. WT, wild-type allele-homozygous females ($n = 16$); HET, heterozygous females ($n = 9$); KO, homozygous AFP KO females ($n = 7$); KO ATD, prenatally ATD-treated AFP KO females ($n = 7$). Differences between values for KO and ATD KO mice in the pituitary are significant ($P \leq 0.044$), while differences between values for HET and ATD KO mice are not ($P \geq 0.508$), except for *Isp2* ($P = 0.014$). For *Gnrh1*, WT-HET differences are not significant, while KO-KO ATD and KO ATD-HET differences are significant (P, 0.049 and 0.002, respectively).

tested: females of both genotypes were fertile and gave birth to litters of normal size. Furthermore, three of these AFP KO females and five of the heterozygous ones were then mated again to determine whether additional litters could be obtained. Females of both genotypes were fertile again and gave birth to litters of normal size comprising both sterile AFP KO and fertile heterozygous females. The correction of the sterility of the treated AFP KO females was thus not transmitted, as expected.

As controls, heterozygous females were injected with propylene glycol only (no ATD). They gave birth to sterile AFP KO and fertile heterozygous females.

(ii) Gene expression. Since prenatal ATD treatment restored the fertility of the AFP KO females, we then asked whether this treatment also normalized the gene expression level in the hypothalamic-pituitary axis of these mice. We tested the expression of several genes in the pituitary, i.e., *Ucp1* (uncoupling protein 1), *Isp2* (implantation serine protease 2), *Egr1* (early growth response 1), and *Gnrhr* (GnRH receptor). The results are shown in Fig. 2. Wild-type and heterozygous animals were different from those used for microarray analysis but were of the same backcross level as the ATD-treated and untreated AFP KO mice. Prenatally ATD-treated AFP KO females exhibited expression levels that were statistically different from those of the untreated KO females ($P \leq 0.044$) but not from those of the heterozygous, untreated females ($P \geq 0.508$), except for the *Isp2* gene in which prenatally ATD-treated KO females are statistically different from heterozygous untreated females. Thus, expression of these genes reached the levels of the fertile groups again. We then tested *Gnrh1* gene expression in the hypothalamus. Surprisingly, the

Gnrh1 expression level in the prenatally ATD-treated AFP KO mice was not increased (Fig. 2).

DISCUSSION

We previously demonstrated that AFP is involved in female fertility, via the activity of the hypothalamic-pituitary axis (21). Taking into account this result, a critical property of AFP is its capacity to bind estrogens (7, 33, 35, 44). Whether this binding reflects a passive, neuroprotective role or a carrier activity has remained a matter of debate (18, 41). The AFP KO mouse allowed us to clarify this issue. Indeed, we show here that fertility of AFP KO female mice is restored after development in an estrogen-free environment; in other words, AFP KO female mice are sterile as a result of prenatal overexposure to estrogen. This result shows that AFP has a merely passive, neuroprotective role. However, the fact that aromatase knockout females are sterile (20) and remain unable to ovulate even after adult estradiol treatment (40) points out that more than prenatal estrogen shielding of the brain alone is needed to achieve normal female reproductive function in adulthood and that estrogen exposure in the postnatal period is necessary for further sexual differentiation.

We also investigated the molecular consequences of prenatal overexposure to estrogen (caused by the absence of embryonic AFP) in the developing brain. We showed that adult AFP KO female mice suffer from anomalies in the levels of numerous gene transcripts. In particular, the GnRH pathway is down-regulated. Our results are in accordance with in vitro studies showing that cellular differentiation and migration of cultured GnRH cells are inhibited if the cultures are exposed to AFP antibodies (17). The changes in gene expression detected in the pituitary may well not be caused directly by prenatal overexposure to estrogen but may be secondary to upstream defects, having occurred in the brain and in particular in sexually dimorphic regions of the hypothalamus. In agreement with this hypothesis, Bakker and coworkers (3) recently showed that AFP KO females have decreased (i.e., male-like) numbers of tyrosine-hydroxylase-immunoreactive cells in the anteroventricular nucleus of the preoptic region.

The fact that the GnRH pathway is stably disturbed while the other main hormonal secretions of the pituitary are unaffected is in accordance with the phenotype of the AFP KO mice in which anovulation is the only phenotypic anomaly detected. Correct integration of the hypothalamic GnRH surge through its pituitary receptor is responsible for the preovulatory luteinizing-hormone surge and ultimately for ovulation. In this respect, it is noticeable that *Egr1* knockout females (28) have many phenotypic similarities to AFP KO females. EGR1 is a transcription factor that binds the *Lhb* promoter and is down-regulated in AFP KO female mice. *Lhb* mRNA levels are not affected in female AFP KO mice, but since these mice do not cycle properly, they could not be analyzed in the proestrus phase of the sexual cycle in which the luteinizing-hormone surge occurs; consequently, the *Lhb* mRNA levels we measured are basal levels, which thus appear to be normal.

AFP KO male mice show no differences in *Gnrh1*, *Gnrhr*, or *Fsh* gene expression, which is consistent with their normal phenotype. Thus, the absence of embryonic AFP seems to interfere with female brain development only.

Prenatal treatment of AFP KO female mice with an aromatase inhibitor not only rescues fertility but also restores the expression profile of the tested genes in the pituitary to values similar to those of normal, fertile heterozygous females. This result strengthens the correlation between abnormal gene expression in the hypothalamic-pituitary axis and female fertility, pointing to a causal relationship between the molecular anomalies and the phenotypic defect. However, heterozygous females, while fertile, show a GnRH receptor gene expression level intermediate between those of WT and AFP KO females, pointing out an AFP dose-dependent effect. Unexpectedly, in the hypothalamus, the expression of the *Gnrh1* gene remains abnormally low, while the level of pituitary *Gnrhr* mRNA is normalized. As *Gnrh1* is a highly regulated gene, with transcriptional and posttranscriptional regulations (for a review, see reference 22), it is possible that stabilization of the decapeptide occurs, compensating for the low mRNA level. GnRH is capable of regulating its own secretion by ultrashort feedback mechanisms mediated by GnRH receptors present in a subpopulation of GnRH neurons (46). On the other hand, since pulsatility of the GnRH action is a critical feature (14), another explanation might be that in ATD-treated AFP KO females, even suboptimal levels of GnRH could elicit an adequate pituitary response provided they are delivered in the right pulsatile manner.

Anovulation caused by a dysfunction of the hypothalamic-pituitary axis is frequently observed in women consulting for fertility issues. Children with a congenital absence of AFP due to a frameshift mutation in the eighth exon of the gene have been described (37), and it will be interesting to monitor the fertility and hormonal levels of women homozygous for the mutation.

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REFERENCES

1. **Abelev, G. I., I. V. Assecritova, N. A. Kraevsky, S. D. Perova, and N. I. Perevodchikova.** 1967. Embryonal serum alpha-globulin in cancer patients: diagnostic value. Int. J. Cancer **2:**551–558.
2. **Andrews, G. K., M. Dziadek, and T. Tamaoki.** 1982. Expression and methylation of the mouse alpha-fetoprotein gene in embryonic, adult, and neoplastic tissues. J. Biol. Chem. **257:**5148–5153.
3. **Bakker, J., C. De Mees, Q. Douhard, J. Balthazart, P. Gabant, J. Szpirer, and C. Szpirer.** Alpha-fetoprotein protects the developing female mouse brain from masculinization and defeminization by estrogens. Nat. Neurosci., in press.
4. **Baldi, P., and A. D. Long.** 2001. A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes. Bioinformatics **17:**509–519.
5. **Benjamini, Y., and D. Yekutieli.** 2001. The control of the false discovery rate in multiple testing under dependency. Ann. Stat. **29:**1165–1188.
6. **Benjamini, Y., and Y. Hochberg.** 1995. Controlling the false discovery rate. A practical and powerful approach to multiple testing. J. R. Stat. Soc. B **57:** 289–300.
7. **Bennett, J. A., F. B. Mesfin, T. T. Andersen, J. F. Gierthy, and H. I. Jacobson.** 2002. A peptide derived from alpha-fetoprotein prevents the growth of estrogen-dependent human breast cancers sensitive and resistant to tamoxifen. Proc. Natl. Acad. Sci. USA **99:**2211–2215.
8. **Brand, T., J. Kroonen, J. Mos, and A. K. Slob.** 1991. Adult partner prefer-

ence and sexual behavior of male rats affected by perinatal endocrine manipulations. Horm. Behav. **25:**323–341.
9. **Cargill, S. L., J. F. Medrano, and G. B. Anderson.** 1999. Infertility in a line of mice with the high growth mutation is due to luteal insufficiency resulting from disruption at the hypothalamic-pituitary axis. Biol. Reprod. **61:**283–287.
10. **Cheng, K. W., and P. C. Leung.** 2000. The expression, regulation and signal transduction pathways of the mammalian gonadotropin-releasing hormone receptor. Can. J. Physiol. Pharmacol. **78:**1029–1052.
11. **Chiu, J. F., D. P. Huang, A. L. Burkhardt, G. Cote, and C. E. Schwartz.** 1983. The alteration of gene expression in rat liver during chemical carcinogenesis. Arch. Biochem. Biophys. **222:**310–320.
12. **Clayton, R. N.** 1988. Mechanism of GnRH action in gonadotrophs. Hum. Reprod. **3:**479–483.
13. **Clayton, R. N., A. R. Solano, A. Garcia-Vela, M. L. Dufau, and K. J. Catt.** 1980. Regulation of pituitary receptors for gonadotropin-releasing hormone during the rat estrus cycle. Endocrinology **107:**699–706.
14. **Conn, P. M., D. C. Rogers, and S. G. Seay.** 1984. Biphasic regulation of the gonadotropin-releasing hormone receptor by receptor microaggregation and intracellular Ca2+ levels. Mol. Pharmacol. **25:**51–55.
15. **Conn, P. M., and W. F. Crowley, Jr.** 1994. Gonadotropin-releasing hormone and its analogs. Annu. Rev. Med. **45:**391–405.
16. **Crowley, W. R., and S. P. Kalra.** 1994. Neonatal exposure to estradiol prevents the expression of ovarian hormone-induced luteinizing hormone and prolactin surges in adulthood but not antecedent changes in neuropeptide Y or adrenergic transmitter activity: implications for sexual differentiation of gonadotropin secretion. Brain Res. **663:**257–265.
17. **Daikoku, S., and I. Koide.** 1994. In vitro development of placode-derived LHRH neurons: possible involvement of alpha-fetoprotein. Horm. Behav. **28:**328–335.
18. **Dohler, K. D.** 1991. The pre- and postnatal influence of hormones and neurotransmitters on sexual differentiation of the mammalian hypothalamus. Int. Rev. Cytol. **131:**1–57.
19. **Duan, W. R., M. Ito, Y. Park, E. T. Maizels, M. Hunzicker-Dunn, and J. L. Jameson.** 2002. GnRH regulates early growth response protein 1 transcription through multiple promoter elements. Mol. Endocrinol. **16:**221–233.
20. **Fisher, C. R., K. H. Graves, A. F. Parlow, and E. R. Simpson.** 1998. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc. Natl. Acad. Sci. USA **95:**6965–6970.
21. **Gabant, P., L. Forrester, J. Nichols, T. Van Reeth, C. De Mees, B. Pajack, A. Watt, J. Smitz, H. Alexandre, C. Szpirer, and J. Szpirer.** 2002. Alpha-fetoprotein, the major fetal serum protein, is not essential for embryonic development but is required for female fertility. Proc. Natl. Acad. Sci. USA **99:**12865–12870.
22. **Gore, A. C., and J. L. Roberts.** 1997. Regulation of gonadotropin-releasing hormone gene expression in vivo and in vitro. Front. Neuroendocrinol. **18:**209–245.
23. **Gorski, R. A.** 1985. Sexual dimorphisms of the brain. J. Anim. Sci. **61:**38–61.
24. **Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249–264.
25. **Irizarry, R. A., L. Gautier, and L. Cope.** 2003. An R package for oligonucleotide array statistical analysis, p. 450–455. *In* G. Parmigiani, E. S. Garrett, R. Irizarry, and S. L. Zeger (ed.), The analysis of gene expression data: methods and software. Springer, New York, N.Y.
26. **Jones, E. A., M. Clement-Jones, O. F. James, and D. I. Wilson.** 2001. Differences between human and mouse alpha-fetoprotein expression during early development. J. Anat. **198:**555–559.
27. **Labdenne, P., and M. Heikinheimo.** 2002. Clinical use of tumor markers in childhood malignancies. Ann. Med. **34:**316–323.
28. **Lee, S. L., Y. Sadovsky, A. H. Swirnoff, J. A. Polish, P. Goda, G. Gavrilina, and J. Milbrandt.** 1996. Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). Science **273:**1219–1221.
29. **MacLusky, N. J., and F. Naftolin.** 1981. Sexual differentiation of the central nervous system. Science **211:**1294–1302.
30. **Marian, J., R. L. Cooper, and P. M. Conn.** 1981. Regulation of the rat pituitary gonadotropin-releasing hormone receptor. Mol. Pharmacol. **19:**399–405.
31. **Masopust, J., K. Kithier, J. Radl, J. Koutecky, and L. Kotal.** 1968. Occurrence of fetoprotein in patients with neoplasms and non-neoplastic diseases. Int. J. Cancer **3:**364–373.
32. **Mizejewski, G. J.** 1985. New insights into AFP structure and function: potential biomedical applications, p. 5–34. *In* G. J. Mizejewski and I. H. Porter (ed.), Alpha-fetoprotein and congenital disorders. Academic Press, Orlando, Fla.
33. **Nishi, S., H. Matsue, H. Yoshida, R. Yamaoto, and M. Sakai.** 1991. Localization of the estrogen-binding site of alpha-fetoprotein in the chimeric human-rat proteins. Proc. Natl. Acad. Sci. USA **88:**3102–3105.
34. **Osada, T., G. Watanabe, S. Kondo, M. Toyoda, Y. Sakaki, and T. Takeuchi.** 2001. Male reproductive defects caused by puromycin-sensitive aminopeptidase deficiency in mice. Mol. Endocrinol. **15:**960–971.
35. **Savu, L., C. Benassayag, G. Vallette, N. Christeff, and E. Nunez.** 1981. Mouse alpha 1-fetoprotein and albumin. comparison of their binding properties with estrogen and fatty acid ligands. J. Biol. Chem. **256:**9414–9418.
36. **Schaapveld, R. Q., J. T. Schepens, G. W. Robinson, J. Attema, F. T. Oerlemans, J. A. Fransen, M. Streuli, B. Wieringa, L. Hennighausen, and W. J. Hendriks.** 1997. Impaired mammary gland development and function in mice lacking LAR receptor-like tyrosine phosphatase activity. Dev. Biol. **188:**134–146.
37. **Sharony, R., I. Zadik, and R. Parvari.** 2004. Congenital deficiency of alpha feto-protein. Eur. J. Hum. Genet. **12:**871–874.
38. **Sukhatme, V. P., X. Cao, L. C. Chang, C. H. Tsai-Morris, D. Stamenkovich, P. C. P. Ferreira, D. R. Cohen, S. A. Edwards, T. B. Shows, T. Curran, M. M. Le Beau, and E. D. Adamson.** 1988. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell **53:**37–43.
39. **Tilghman, S. M., and A. Belayew.** 1982. Transcriptional control of the murine albumin/alpha-fetoprotein locus during development. Proc. Natl. Acad. Sci. USA **79:**5254–5257.
40. **Toda, K., K. Takeda, T. Okada, S. Akira, T. Saibara, T. Kaname, K. Yamamura, S. Onishi, and Y. Shizuta.** 2001. Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17beta-oestradiol. J. Endocrinol. **170:**99–111.
41. **Toran-Allerand, C. D.** 1984. On the genesis of sexual differentiation of the general nervous system: morphogenetic consequences of steroidal exposure and possible role of alpha-fetoprotein. Prog. Brain Res. **61:**63–98.
42. **Tremblay, J. J., and J. Drouin.** 1999. Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone β gene transcription. Mol. Cell. Biol. **19:**2567–2576.
43. **Uriel, J., D. Bouillon, C. Aussel, and M. Dupiers.** 1976. Alpha-fetoprotein: the major high-affinity estrogen binder in rat uterine cytosols. Proc. Natl. Acad. Sci. USA **73:**1452–1456.
44. **Vakharia, D., and G. J. Mizejewski.** 2000. Human alpha-fetoprotein peptides bind estrogen receptor and estradiol, and suppress breast cancer. Breast Cancer Res. Treat. **63:**41–52.
45. **Wurmbach, E., T. Yuen, B. J. Ebersole, and S. C. Sealfon.** 2001. Gonadotropin-releasing hormone receptor-coupled gene network organization. J. Biol. Chem. **276:**47195–47201.
46. **Xu, C., X. Z. Xu, C. S. Nunemaker, and S. M. Moenter.** 2004. Dose-dependent switch in response of gonadotropin-releasing hormone (GnRH) neurons to GnRH mediated through the type I GnRH receptor. Endocrinology **145:**728–735.
47. **Xu, Z. P., E. F. Wawrousek, and J. Piatigorsky.** 2002. Transketolase haploinsufficiency reduces adipose tissue and female fertility in mice. Mol. Cell. Biol. **22:**6142–6147.